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Cdc13 Cooperates with the Yeast Ku Proteins and Stn1 To Regulate Telomerase Recruitment

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The *Saccharomyces cerevisiae CDC13* protein binds single-strand telomeric DNA. Here we report the isolation of new mutant alleles of *CDC13* that confer either abnormal telomere lengthening or telomere shortening. This deregulation not only depended on telomerase (Est2/*TLC1*) and Est1, a direct regulator of telomerase, but also on the yeast Ku proteins, yKu70/Hdf1 and yKu80/Hdf2, that have been previously implicated in DNA repair and telomere maintenance. Expression of a Cdc13-yKu70 fusion protein resulted in telomere elongation, similar to that produced by a Cdc13-Est1 fusion, thus suggesting that yKu70 might promote Cdc13-mediated telomerase recruitment. We also demonstrate that Stn1 is an inhibitor of telomerase recruitment by Cdc13, based both on *STN1* overexpression and Cdc13-Stn1 fusion experiments. We propose that accurate regulation of telomerase recruitment by Cdc13 results from a coordinated balance between positive control by yKu70 and negative control by Stn1. Our results represent the first evidence of a direct control of the telomerase-loading function of Cdc13 by a double-strand telomeric DNA-binding complex.

Telomeres, the ends of eukaryotic chromosomes, are critical for maintaining chromosome stability and genome integrity (2, 8, 60). Telomeres are composed of particular DNA sequences which are rich in TG and arranged in species-specific repeated motifs. Telomeres are capped by proteins that bind to these repeating DNA sequences (6, 20). This apparently serves at least two distinct purposes. First, some of these telomeric proteins presumably form complexes that regulate telomerase activity and, hence, the length of telomeric tracts (31, 43). Some telomeric proteins have also been implicated in the physical protection of chromosome ends (38), in preventing recombinational events that would otherwise frequently occur between repeating telomeric sequences (33, 34, 53), and in keeping off DNA repair enzymes (14). Indeed, telomeres represent naturally occurring DNA double-strand breaks that, contrary to those resulting from accidental damage, do not need to (and must not) be repaired. Surprisingly, however, yeast Ku proteins, as well as proteins of the Mre11-Rad50-Xrs2 complex, which have been implicated in DNA repair by nonhomologous end joining have also been implicated in telomere maintenance (3, 4, 7, 11, 12, 27, 28, 39, 44, 46, 47). Moreover, yKu70 and yKu80 have been found to localize at the telomeres (18, 37).

The repeating TG-rich telomeric DNA sequences are mostly double stranded. However, during S phase only, telomeres display a short (ca. 35- to 50-nucleotide) single-stranded DNA extension that marks the very end of the telomere (57, 58). Single-stranded telomeric DNA is thought to represent the site of anchoring of telomerase, which is composed of the evolutionary conserved Est2 reverse transcriptase enzyme and of the *TLC1* RNA template (31, 42). However, recent experiments suggest that telomerase-dependent elongation of de novo ends does not appear to involve single strandedness and does not require significant degradation prior to addition of newly synthesized telomeric DNA (9). Est1 and Est3 represent two subunits of the telomerase complex (25, 29, 55), which although not required for in vitro telomerase catalytic activity (32), are nevertheless stable components of the enzyme and regulate its activity in vivo through physical association with Est2 and *TLC1* (25, 61).

Cdc13 was the first identified single-strand DNA-binding telomeric protein in *Saccharomyces cerevisiae* and, consequently, its status as a candidate for most of telomeric functions has become prominent (5, 14, 30, 45). The isolation of the *cdc13-2*/*est4-1* allele, which confers a strong deficit in telomerase activity (29), as well as the recent finding that a fusion protein made of Cdc13 plus Est1 could bypass telomerase-defective mutations in either protein, strongly suggests that interactions between Cdc13 and Est1 represent the mechanism by which a number of regulators can control telomerase recruitment (10). Indeed, physical association between Cdc13 and Est1 has been revealed recently (48). Cdc13 has also been shown to bind Pol1 in vivo, and it has been proposed that Cdc13 might coordinate regulation at the telomere ends of G-strand lengthening by telomerase, via Est1, and C-strand resynthesis by polymerase α (48). In addition, the observation that the temperature-sensitive *cdc13-1* allele displays abnormal accumulation of single-stranded DNA specifically at telomeric regions of chromosomes argued that Cdc13 might be a major telomeric capping protein (14). It has also been observed that when Cdc13 was defective, in *cdc13-1* cells, the absence of either yKu70 or yKu80, which was otherwise dispensable, impaired growth (44, 46). The yKu proteins, which bind to double-stranded telomeric DNA, have been proposed to be involved in establishing the proper terminal DNA structure of chromosomes in cooperation with telomerase (18, 46). In addition to its nonessential function in the recruitment of telomerase at telomere ends (45), Cdc13 has an essential function that has not yet been clearly defined. *cdc13-1* mutant cells are temperature sensitive and present a first cell cycle arrest at restrictive temperatures of growth (14).

In the present study, we have isolated several new mutant alleles of *CDC13* which confer either abnormal telomere elongation or, on the contrary, telomere shortening. Telomere elongation in these novel *cdc13* alleles was found to be more affected by mutations in either *YKU70* or *YKU80* than by mutations in *TEL1* or *RAD50*, therefore implicating the yeast Ku proteins in the telomerase-loading function of Cdc13. This was

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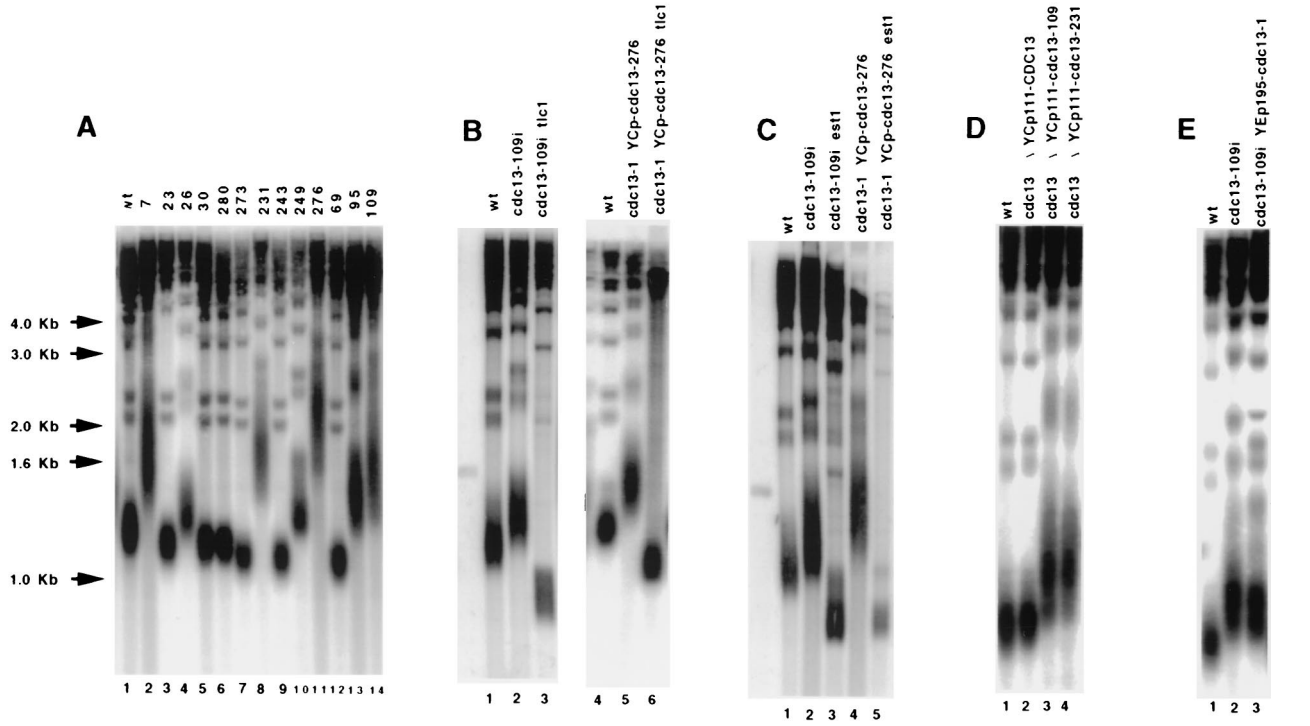

FIG. 1. Telomere lengthening associated with the *cdc13* alleles relies on telomerase activity and requires Est1. (A) Telomere Southern blot analysis of novel non-temperature-sensitive *cdc13* mutant alleles generated by PCR mutagenesis (see Materials and Methods) reveals the possibility of a large panel of telomere size deregulation when *CDC13* is mutated (lanes 2 to 14) compared to a wild-type strain (lane 1). Here, the *cdc13* alleles (names of which are indicated on top of the figure) were present on a single-copy centromeric plasmid in a *cdc13-1* mutant. These mutant strains were grown at 32°C to inactivate Cdc13-1. (B) Augmentation of telomerase activity is responsible for telomere elongation in the new *cdc13* mutant strains described here. *TLC1*, the RNA subunit of telomerase, was disrupted directly in a *cdc13-109i* mutant or in a *cdc13-1* mutant harboring YCp-*cdc13-276*. The resulting strains no longer displayed the telomere elongation conferred by the *cdc13-109* and *cdc13-276* mutations, as measured here after approximately 50 generations (compare lane 2 to lane 3 and lane 5 to lane 6). Telomere lengths in wild-type strains (lanes 1 and 4) are shown for comparison. (C) Telomere elongation in the *cdc13* mutants necessitates the presence of Est1, a regulator of telomerase. The *EST1* gene was genetically disrupted in *cdc13-109i* or in *cdc13-1* YCp-*cdc13-276* and telomere size measured after approximately 50 generations. Absence of telomere lengthening in the resulting double mutants (compare lane 2 to lane 3 and lane 4 to lane 5) was observed. (D) The Cdc13-109 and Cdc13-231 mutant proteins can sustain growth on their own when expressed from a single-copy plasmid in a strain disrupted for *CDC13* (*cdc13*::*TRP1*). Both Cdc13-109 (lane 3) and Cdc13-231 (lane 4) conferred telomere lengthening compared to a wild-type strain (lane 1) or a *cdc13* disruptant expressing wild-type *CDC13* (lane 2). (E) Expression of the temperature-sensitive *cdc13-1* allele from an episomal plasmid, at the restrictive temperature of 34°C, had no incidence on the *cdc13-109*-associated telomere lengthening (compare lanes 2 and 3).

supported by observing telomere elongation as a direct result of the expression of a Cdc13-yKu70 fusion protein, which is comparable in length to that produced by a Cdc13-Est1 fusion. We also present evidence, based on overexpression of Stn1 or expression of Stn1-Cdc13 fusions, that Stn1, a protein that associates with Cdc13 by two-hybrid interaction (17), is an inhibitor of telomerase recruitment via Cdc13. We propose that Cdc13 is both a positive and a negative regulator of telomerase recruitment, which establishes differential interactions with other telomeric proteins, and that the balance between these two opposing effects principally relies on interactions with yKu70 or yKu80 and with Stn1.

MATERIALS AND METHODS

Strains, plasmids, and media. General plasmids and media used in this study were as described previously (17). Disruptions of *TLC1*, *EST1*, *RAD50*, *YKU70*, or *YKU80* in wild-type, *cdc13-1*, or *cdc13-109i* strains were achieved following transformation of linearized *tlc1*::*LEU2* (52), *est1*::*URA3* (55), *rad50*::*hisG-URA3-hisG* (41), *yku70*/*hdf1*::*URA3* (47), or *yku80*::*TRP1* (see below) DNA fragments. The correct disruptions were detected on Southern blots. In some cases, these disruption strains were further mixed with other mutations by genetic crosses. The *tel1*::*kanMX4* (strain record number 3114; Research Genetics, Inc., Huntsville, Ala.) and *rad52-7*::*LEU2* (strain record number XS560-1C-1D1; Yeast Genetic Stock Center, Berkeley, Calif.) disruption strains were backcrossed five times against the genetic background used in our lab (17). The *cdc13*::*TRP1* disruption plasmid was constructed by inserting *TRP1* at the *Bam*HI site of *CDC13*, at nucleotide 1349, and the *yku80*::*TRP1* disruption plasmid was constructed by inserting *TRP1* between the *Xba*I and *Acc*I sites (nucleotides 84 to 1762) of *YKU80*. The *cdc13-109* integrated allele was constructed by the pop-in–pop-out method. To do this, *cdc13-109* was cloned into a *URA3* integrative plasmid (YIp211) which was then used to transform a wild-type strain. The Ura^+ transformants were then grown on 5-fluoro-orotic acid (5-FOA) plates to counterselect for cells that, together with the *URA3* marker gene, had lost one copy of the *CDC13* gene (either the wild-type or the mutant copy). Telomere length was then monitored on Southern blots in several of these Ura^- cells after a few generations of growth (see below). Only half of these colonies displayed elongated telomeres, with the other half exhibiting telomeres of wild-type size. Cells with elongated telomeres were selected, and the presence of only one copy of the *CDC13* gene was verified by Southern blot. This suggested that cells with elongated telomeres had integrated the *cdc13-109* allele at the *CDC13* locus, while cells with wild-type telomeres had, on the contrary, evicted the *cdc13-109-URA3* integrated construct. Integration of the *cdc13-69* allele at the *CDC13* locus was performed using the same methodology as for *cdc13-109*.

Construction and selection of *cdc13* alleles and of *stn1-63*. *CDC13* open reading frame (ORF) plus 300 bp upstream of the ATG was amplified by PCR under mutagenic conditions, as described previously for *STN1* (17) in standard PCR buffer supplemented with $MnCl_2$, using standard *Taq* polymerase (Gibco-BRL). Following two rounds of PCR mutagenesis, using the products of the first reaction as a template for the second reaction, the PCR products were cleaned and used directly to transform a *cdc13-1* strain, together with a single-copy, centromeric, plasmid, YCp111-*LEU2* (15), made linear by digestion and carrying *CDC13* flanking regions at each extremity (gap repair method). The 5′ fragment of these flanking regions consisted of the 964 bases before the start codon plus the 57 bases after, while its 3′ fragment comprised the 690 bases before the stop codon plus the 830 bases after. Cells were then plated onto leucine-lacking (Leu^-) medium and incubated at 25°C until colonies of transformants developed. Because the objective of *CDC13* mutagenesis was to uncover non-temper-

ature-sensitive alleles deregulated in telomere length control, transformants were then replica plated on Leu$^-$ medium at 32 and 34°C, temperatures of growth restrictive for *cdc13-1*. This allowed us to select for mutagenized *CDC13* plasmids capable of sustaining growth of the cells bearing them in the absence of functional endogenous Cdc13 protein, because the Cdc13-1 protein is inactivated at temperatures higher than 28°C in our genetic background (17). Among several thousands of such colonies growing at 32 or 34°C, 121 were picked out randomly and separately grown for further analysis of the length of their telomeric tracts (see below). The most interesting YCp111-borne *cdc13* alleles, in terms of telomere length deregulation, were then recovered from the original *cdc13-1* recipient strain and used to retransform the *cdc13-1* strain. Genomic DNA from transformants grown for about 100 generations was then prepared, and the lengths of the telomeric tracts were analyzed by Southern blotting, as explained below.

The *stn1-63* allele was generated by PCR mutagenesis, followed by gap repair, under conditions described previously (17). The *stn1*::*TRP1* strain bearing the *stn1-63* allele on a YCp111-*LEU2* plasmid was selected (after eviction of the wild-type *STN1* allele carried by a YCp33-*URA3* plasmid on 5-FOA-containing medium) among several tens of other potential *stn1* mutant strains on the basis of telomere length deregulation, as directly measured on Southern blots, as described below.

For sequence analysis of the *cdc13* alleles, the ORFs of the mutant *CDC13* genes, cloned into YEp195-*GAL1* (an episomal, 2μ, *URA3* plasmid), were digested with *Eco*RI, taking advantage of the presence of two natural *Eco*RI sites in the *CDC13* sequence. This generated three pieces of *CDC13* ORF roughly equal in size, which were then subcloned into pBluescript. DNA sequencing was performed in a semiautomated DNA sequencer (Applied Biosystems) using T3 or T7 primer as the sequencing primer.

Measurement of telomere length. Genomic DNA was prepared as described previously (17), digested with *Xho*I and separated by electrophoresis in a 0.9% agarose gel in Tris-borate-EDTA. After denaturation, DNA was transferred onto nitrocellulose membrane and immobilized by baking at 80°C for 1 h under a vacuum (1). The membrane was then prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate–1% nonfat milk and hybridized with a 270-bp TG_{1-3} ^{32}P-labeled probe representing *S. cerevisiae* telomeric sequences. Results were analyzed using a Storm PhosphorImager (Molecular Dynamics).

Control experiments in which DNA fragments after *Xho*I cutting were separated under denaturing conditions (1% agarose gel run in 40 mM NaOH–2 mM EDTA) were performed to ensure that both single-stranded and double-stranded modifications in telomere length were detected (see also reference 17).

Construction of fusion proteins. Cdc13-Est1, Cdc13-69-Est1, Cdc13-yKu70, Cdc13-yKu80, Cdc13-Stn1-63, and Cdc13-231-Stn1 in-frame fusion proteins were constructed by cloning in a single-copy, centromeric, plasmid the entire *CDC13* ORF (or the *cdc13-69* or *cdc13-231* ORFs) plus upstream promoter sequences in front of the *EST1*, *YKU70*, *YKU80*, or *STN1* (wild-type or mutant) ORF (which included their natural stop codons). The yKu80-Cdc13 fusion protein was constructed by cloning in a single-copy plasmid the entire *YKU80* ORF plus upstream promoter sequences in front of *CDC13*, so as to keep a continuous reading frame. The *CEN* plasmids used in the present study are of the YCplac series and are single-copy plasmids (15), just like the *CEN* plasmid, pRS415 (51), used by Evans and Lundblad (10). Telomere elongation by the Cdc13-Est1 fusion protein was not due to increased protein expression because overexpression of *CDC13* or *EST1* had no effect on telomere length (10, 17). Moreover, the functionality of the Cdc13-Est1 fusion protein was attested to by its ability to assume the essential function of Cdc13 (rescue of *cdc13-1* cells at a restrictive temperature or of *cdc13*Δ cells) and to rescue the senescence phenotype of an *est1*::*URA3* mutant.

Epitope tagging, Western blot detection, and band shift experiments. The 10 *cdc13* mutant DNAs corresponding to the mutant strains described in the present study were subcloned into a YEp195-*GAL1* (2μ, *URA3*) plasmid under the control of the strong, inducible, *GAL1* promoter. In this plasmid the natural stop codon of the *cdc13* mutant genes was eliminated so as to obtain a continuous reading frame between the Cdc13 protein and a 2HA-6His epitope tag (16). This allowed visualization of the Cdc13 mutant proteins by Western blotting using monoclonal anti-hemagglutinin antibody (12CA5; Boehringer). The presence of this epitope tag also allowed purification of the Cdc13 mutant proteins by Ni chromatography directed against the His_6 part of the tag, using Qiagen reagents. After transfer to nitrocellulose membrane (Schleicher & Schuell), proteins were detected using an enhanced chemiluminescence system (ECF; Amersham) coupled with detection in a Storm PhosphorImager (Molecular Dynamics).

For band shift experiments and detection by Western blotting, wild-type cells bearing, on a YEp195-*GAL1-URA3* plasmid, wild-type *CDC13* or a *cdc13* mutant allele fused in 3′ with a 2HA-6His epitope were grown overnight, in liquid cultures, in glucose-based minimal medium lacking uracil. Cdc13 protein expression was then induced for 4 h at 30°C after shifting the cells to Ura$^-$ galactose-containing medium following four or five washes in galactose-based medium. Cells were then harvested by centrifugation, and extracts were prepared in band shift lysis buffer (50 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 8.0; 30 mM NaCl; 10 mM Na imidazole) supplemented with protease inhibitors (1% phenylmethylsulfonyl fluoride and 10 μg each of aprotinin, leupeptin, and pepstatin per ml). Approximately 500 μg of the total proteins were then incubated with Ni-nitrilotriacetic acid beads (Qiagen) for 2 h at 4°C. Nickel beads were then washed three times in 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0), 20 mM Na imidazole, and 0.5% Tween 20 and then once in 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0) and 20 mM Na imidazole. Cdc13–2HA-6His proteins were then eluted in 50 μl of 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0)–250 mM Na imidazole. Binding reactions were performed in 50 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 1 μg of single-stranded poly(dI-dC) using 2 to 4 μl of eluted protein, and 1 ng of ^{32}P-labeled $(TG_{1-3})_3$ (TGTGTGGGTGTGTG GGTGTGTGGG) for 20 min at 30°C. A monoclonal anti-HA antibody (12CA5) was used at 0.5 μg per reaction. After the addition of 1 μl of 10% glycerol, the reactions were loaded on a 4.5% polyacrylamide gel that was run for 2 h at 4°C. Gels were then dried and analyzed using a Storm PhosphorImager (Molecular Dynamics).

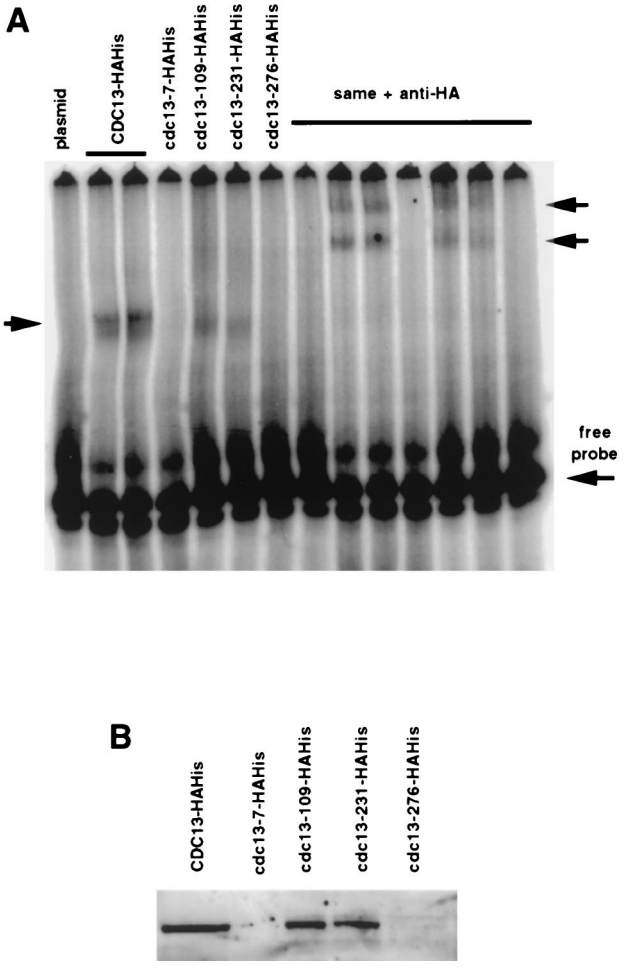

FIG. 2. The telomere-elongating Cdc13 mutant proteins described in this study are still able to bind telomeric sequences. (A) The in vitro binding of wild-type Cdc13 and of telomere-elongating Cdc13 mutant proteins to specific TG_{1-3} telomeric DNA sequences was measured by assessing gel retardation of Cdc13-2HA-6His proteins (see Materials and Methods). The same reaction mixtures were incubated in parallel with a monoclonal anti-HA antibody, which generated a supershift of the complex attesting of the specificity of the DNA-protein binding reaction. Binding of the Cdc13-109–2HA–6His and Cdc13-231–2HA–6His proteins was somewhat weaker than that of wild-type Cdc13-2HA-6His, while Cdc13-7–2HA–6His and Cdc13-276–2HA–6His did not bind telomeric DNA. Unlabeled arrows indicate the position of the DNA-protein and DNA-protein-antibody complexes. (B) Immunoblot analysis, using a monoclonal anti-HA antibody, of crude extracts from cells expressing Cdc13-2HA-6His proteins under the control of the inducible *GAL1* promoter was performed essentially to assess the presence of the Cdc13 mutant proteins used in band shift experiments. This analysis revealed that, in fact, Cdc13-7 and Cdc13-276 were not produced as entire proteins. Sequence analysis of these *cdc13* alleles revealed the presence of a stop codon, generated during mutagenesis, upstream of the natural stop codon (see text).

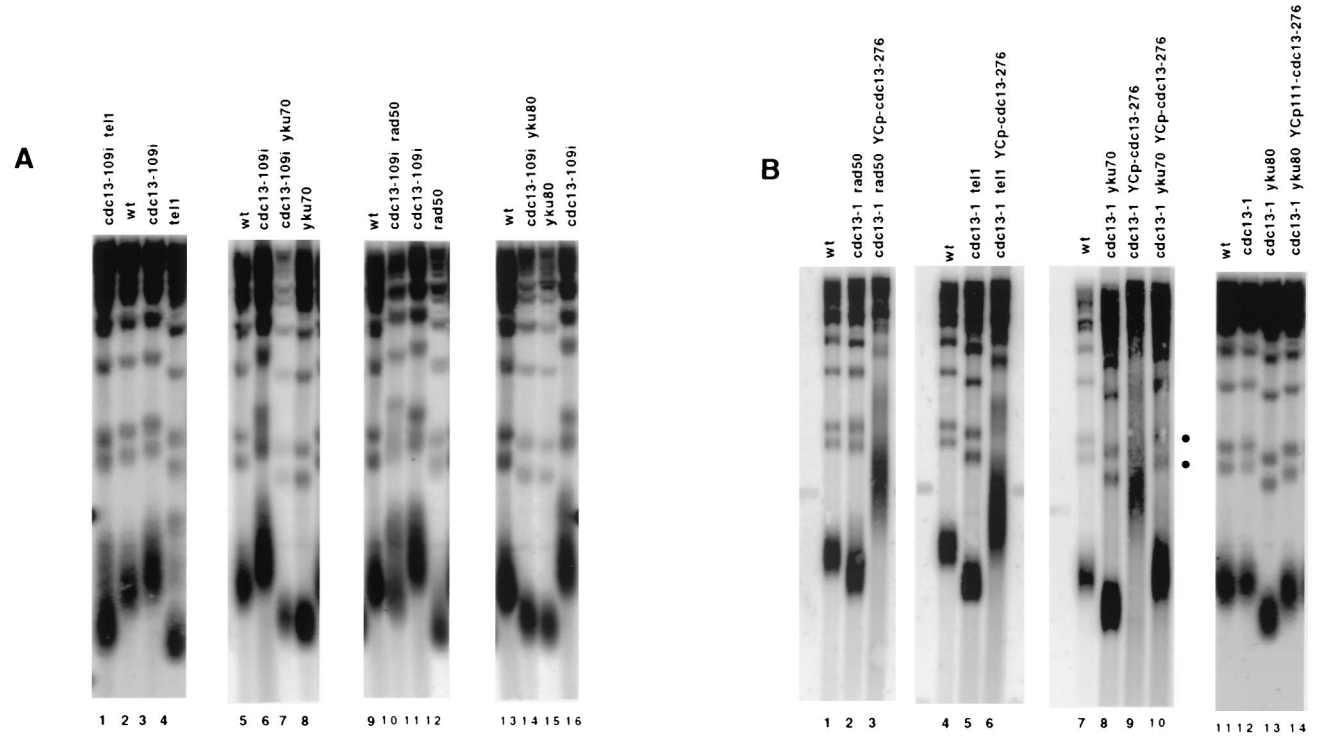

FIG. 3. Mutations in *YKU70* or *YKU80* have a larger suppressing effect on *cdc13-109*- or *cdc13-276*-associated telomere elongation than mutations in *RAD50* or *TEL1*. (A) *cdc13-109i tel1* (lane 1), *cdc13-109i yku70* (lane 7), *cdc13-109i rad50* (lane 10), and *cdc13-109i yku80* (lane 14) double mutants were grown for approximately 100 generations, and their telomere lengths were compared to those in the corresponding single mutants (neighboring lanes in each panel for each of the four mutations) and in wild-type cells (lanes 2, 5, 9, and 13). See the text for interpretation of the data. (B) *cdc13-1 rad50*Δ, *cdc13-1 tel1*Δ, *cdc13-1 yku70*Δ, and *cdc13-1 yku80*Δ double mutants were propagated at 25°C and transformed with a single-copy plasmid harboring the *cdc13-276* allele. The resulting triple mutants were grown at 34°C to inactivate Cdc13-1, and the sizes of their telomeres were measured after approximately 100 generations (lanes 3, 6, 10, and 14) and compared to those in *cdc13-1 rad50*, *cdc13-1 tel1*, *cdc13-1 yku70*, or *cdc13-1 yku80* (lanes 2, 5, 8, and 13), respectively, at 34°C or to that in wild-type isogenic strains (lanes 1, 4, 7, and 11). Telomere lengths in *cdc13-1* cells grown at 25°C (lane 12) and in *cdc13-1* cells harboring *cdc13-276* on a single-copy plasmid, grown at 34°C (lane 9), served as negative and positive controls, respectively. Note that *cdc13-1 yku70*Δ YCp-*cdc13-276* (lane 10) and *cdc13-1 yku80*Δ YCp-*cdc13-276* cells (lane 14) no longer displayed the long telomere phenotype characteristic of the *cdc13-276* allele (lane 9), contrary to *cdc13-1 rad50* YCp-*cdc13-276* (lane 3) and *cdc13-1 tel1* YCp-*cdc13-276* (lane 6) which did. This interpretation is confirmed by visualizing the dots in lane 10, which highlight two bands corresponding to two non Y′ telomeres. These were also present in wild-type cells (lane 7) and *yku70*Δ cells (lane 8) but were absent from cells harboring the *cdc13-276* mutation alone (lane 9). This confirmed that the telomere structure in the *cdc13-1* YCp-*cdc13-276 yku70*Δ mutant no longer resembled that in *cdc13-1* YCp-*cdc13-276* but was instead similar to that in wild-type cells. The same held true for *yku80*Δ (lane 14) but not for *rad50*Δ (lane 3) and *tel1*Δ (lane 6) cells. See the text for further explanations. Conditions of strain culturing were as described above.

RESULTS

New telomere-shortening and telomere-elongating *cdc13* alleles. To better understand the role of Cdc13 at telomeres, we set out to generate new alleles of *CDC13* using PCR amplification under mutagenic conditions (see Materials and Methods) and reintroduce them into a conditional system provided by a *cdc13-1* mutant strain (14). *cdc13-1* cells stop growing at temperatures above 23 to 25°C (14) or above 27 to 28°C in our genetic background (17). Among several thousand transformants of *cdc13-1* capable of growth at 32 or 34°C, 121 were picked randomly for further study. Genomic DNA was prepared, and the lengths of the telomeric tracts were measured by probing total *Xho*I-digested genomic DNA with a ^{32}P-labeled TG_{1-3} telomeric probe, as described in Materials and Methods. Only mutant strains with substantial changes in telomere length were selected for further analysis, namely, *cdc13-7*, *cdc13-109*, *cdc13-231*, and *cdc13-276*, which are telomere-elongating alleles, and *cdc13-23*, *cdc13-30*, *cdc13-69*, *cdc13-243*, *cdc13-273*, and *cdc13-280*, which are telomere-shortening alleles (Fig. 1A).

Since we suspected that the Cdc13-1 mutant protein might have some residual activity at 32 to 34°C, we next tested whether the isolated *cdc13* alleles were capable of sustaining growth on their own. To this end, we attempted to replace the genomic copy of *CDC13* by each one of these *cdc13* alleles using the pop-in–pop-out method (see Materials and Methods). Concerning the telomere-elongating alleles (telomere-shortening alleles will be examined below), we were successful in recovering cells that had integrated a mutant copy of *cdc13-109* (from now on referred to as *cdc13-109i*) at the *CDC13* genomic locus. The Cdc13-109 mutant protein in the *cdc13-109i* strain could assume the essential function of Cdc13 while harboring long telomeres similar to those in the *cdc13-1* strain bearing the *cdc13-109* allele on a centromeric plasmid (Fig. 1A and B). In terms of growth and cellular morphology, the *cdc13-109i* strain was indistinguishable from a wild-type strain (data not shown). We were not successful at obtaining replacements with the other three selected telomere-elongating *cdc13* alleles.

In another approach to characterize these telomere-elongating *cdc13* alleles, we constructed a *cdc13* disruption strain (*cdc13*::*TRP1*), that survived owing to a single-copy *URA3* plasmid expressing wild-type *CDC13*, which was then transformed with one of the four selected *cdc13* alleles carried by a single-copy plasmid. After eviction of the *CDC13-URA3* plasmid on 5-FOA-containing medium, the *cdc13*::*TRP1* YCp-*cdc13-109* and *cdc13*::*TRP1* YCp-*cdc13-231* strains were viable and exhibited telomeres of a length similar to that in the respective

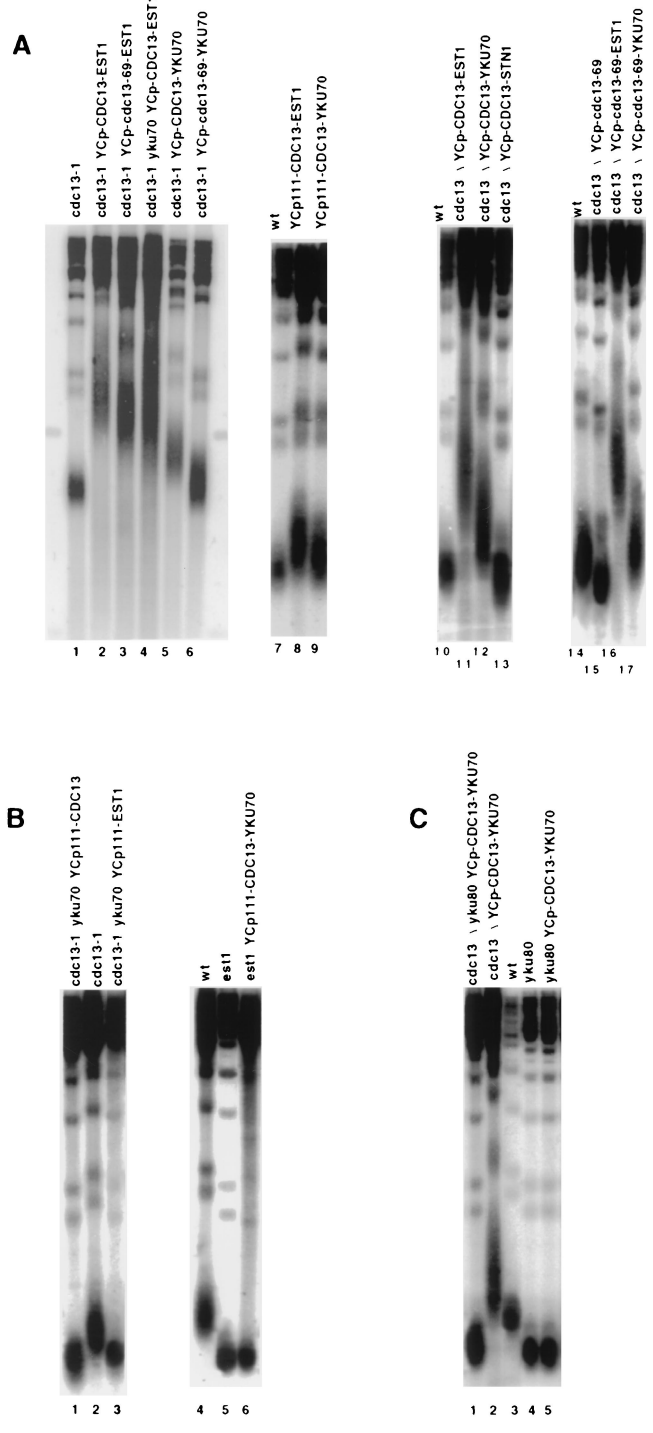

FIG. 4. A Cdc13-yKu70 fusion protein produces telomere elongation that is dependent on Est1. (A) Expression of *CDC13-EST1* or *CDC13-YKU70* hybrid gene from a single-copy centromeric plasmid under the control of the *CDC13* promoter for approximately 100 generations, either in a *cdc13-1* strain grown at 34°C to inactivate Cdc13-1 (left panel), in a wild-type background (middle left panel), or in a *cdc13-1*Δ background (middle right panel) provoked dramatic telomere elongation (lanes 2, 5, 8, 9, 11, and 12) compared to controls (*cdc13-1* cells grown at 25°C, lane 1; wild-type cells, lanes 7 and 10). A fusion consisting of the telomere-shortening Cdc13-69 mutant protein and of either wild-type Est1 or wild-type yKu70 also produced telomere elongation in *cdc13*Δ (lanes 16 and 17) or *cdc13-1* at 34°C (lanes 3 and 6), therefore indicating rescue of the short telomere phenotype conferred by Cdc13-69 (lane 15). A Cdc13-Stn1 fusion (lane 13) served as a negative control. (B) Telomere elongation provoked by the Cdc13-yKu70 fusion no longer took place in the absence of Est1 (lane 6; compare to the *est1*Δ [lane 5] and wild-type [lane 4] strains). Lanes 1 and 3, in which *CDC13* or *EST1* alone were expressed, provided additional controls for these protein fusion experiments. See the text for further explanations. (C) Telomere elongation provoked by the Cdc13-yKu70 no longer took place in *yku80*Δ *cdc13-1*Δ cells (lane 1; compare to the *YKU80*$^+$ *cdc13*Δ cells expressing the same fusion (lane 2) and to the wild-type [lane 3] and *yku80*Δ [lane 4] strains). This effect was observed both in a *cdc13*Δ background (lane 1) and a *CDC13*$^+$ background (lane 5).

original strains in a *cdc13-1* background at 32–34°C (Fig. 1A and D), while the *cdc13*::*TRP1* YCp-*cdc13-7* and the *cdc13*::*TRP1* YCp-*cdc13-276* strains were not viable (data not shown).

To serve as a control for some of the experiments that have been performed in a *cdc13-1* background (see below), we measured telomere length in the *cdc13-109i* strain transformed with *cdc13-1* on an episomal plasmid. Importantly, at 32 or 34°C, telomeres in this strain were the same length as those in the *cdc13-109i* strain (Fig. 1E) and as those in the *cdc13-1* strain harboring *cdc13-109* on a centromeric plasmid (Fig. 1A). These control experiments establish that the presence of the Cdc13-1 mutant protein at 32 to 34°C has no effect on telomere length while, on the other hand, it may provide, at these temperatures, a residual Cdc13 activity allowing growth of strains harboring the *cdc13-7* and *cdc13-276* mutations which are otherwise unable to sustain growth on their own.

Telomere elongation in the new *cdc13* mutants depends on telomerase and requires Est1. Because both telomerase-dependent and telomerase-independent mechanisms, in the latter case relying on homologous recombination, can regulate telomere size (33, 34), we next asked which one of these two mechanisms affected telomere regulation in the telomere-elongating *cdc13* mutants described here. To do this, we introduced a null mutation in *TLC1*, the RNA subunit of telomerase essential for telomerase activity (52), into the *cdc13-109i* mutant (see Materials and Methods) and measured telomere size in the resulting double mutants. The typical telomere elongation observed in the *cdc13-109i* was not observed in the *cdc13-109i tlc1*Δ mutants (Fig. 1B). These results were confirmed using the *cdc13-1* YCp-*cdc13-276* strain (Fig. 1B). Because the homologous recombination mechanisms controlling telomere length are entirely dependent on Rad52, we then analyzed telomere size deregulation in *cdc13-109i rad52*Δ or *cdc13-1* YCp111-*cdc13-276 rad52*Δ mutant strains and found that they displayed telomeres elongated to the same extent as that in the corresponding *RAD52*$^+$ strains (data not shown).

It has been recently demonstrated that Cdc13 regulates telomerase recruitment *via* functional interaction with Est1 (10, 48, 55). We found that telomere elongation associated with the *cdc13-109i* and *cdc13-276* mutations was suppressed in the absence of Est1 (Fig. 1C), implying that Est1 is necessary for abnormal telomere lengthening in the *cdc13-109i* and *cdc13-276* mutants. These results also suggest that regulation of telomerase recruitment by wild-type Cdc13 requires Est1.

The telomere-elongating Cdc13 mutant proteins still associate with telomeric DNA. To determine whether the telomere length phenotype could be merely due to a defect of the Cdc13 mutant proteins in binding telomeric DNA (5, 26, 30), band shift experiments were performed using Cdc13–2HA-6His mutant proteins purified by Ni chromatography (see Materials and Methods). The Cdc13-109 and Cdc13-231 proteins were still be able to bind telomeric DNA (Fig. 2A). The specificity of these interactions was evidenced by visualizing supershifts when a monoclonal anti-HA antibody was added to the reaction (Fig. 2A). Careful examination of the intensities of the DNA-protein bands revealed that the Cdc13-109 and Cdc13-

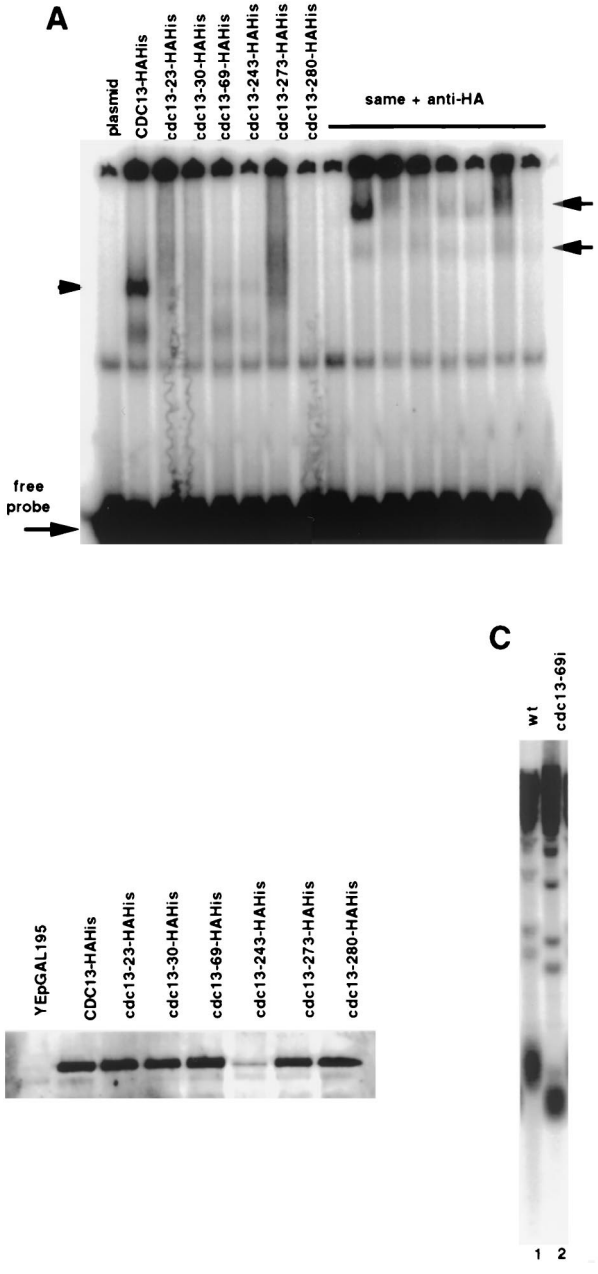

FIG. 5. In vitro binding of telomere-shortening Cdc13 mutant proteins to specific TG_{1-3} telomeric DNA sequences. (A) Band shift experiments, performed as described in the legend to Fig. 2, revealed that all six Cdc13 mutant proteins were severely defective in DNA binding, with Cdc13-23–2HA-6His, Cdc13-30–2HA-6His, and Cdc13-280–2HA-6His being more affected than Cdc13-69–2HA-6His, Cdc13-243–2HA-6His, and Cdc13-273–2HA-6His, which still showed some binding. The specificity of the DNA-protein interaction was attested to by visualizing a supershift upon addition of a monoclonal anti-HA antibody to the reaction mixture. Unlabeled arrows indicate the position of the DNA-protein and DNA-protein-antibody complexes. (B) Western blot analysis of crude extracts from cells expressing Cdc13–2HA-6His proteins under the control of the inducible *GAL1* promoter, using a monoclonal anti-HA antibody, revealed that basically all of the Cdc13 mutant proteins were produced to the same extent within the cell, with the exception of Cdc13-243–2HA-6His, whose levels were lower than those of the other mutant proteins. (C) The *cdc13-69i* strain, harboring the telomere-shortening *cdc13-69* allele integrated at the *CDC13* locus, exhibited telomeres shortened to the same extent as those in a *cdc13-1* YCp-*cdc13-69* strain grown at 34°C (Fig. 1A, lane 12) or in a *cdc13*Δ YCp-*cdc13-69* strain (Fig. 4A, lane 15).

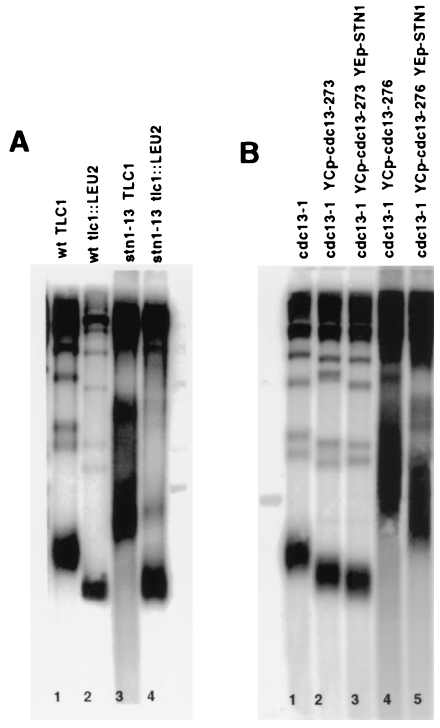

FIG. 6. Stn1 behaves as an inhibitor of telomerase recruitment. (A) A "young" *stn1-13* mutant strain (in which the *stn1-13* mutation had been introduced at the *STN1* locus only for a week so as to obtain cells with telomeres of nearly wild-type size) was disrupted for *TLC1* and grown at 30°C for approximately 50 generations. In this *stn1-13 tlc1*::*LEU2* strain, telomeres remained short (lane 4), of the same size as in a wild-type strain disrupted for *TLC1* (lane 2), and shorter than in wild-type $TLC1^+$ cells (lane 1), in contrast with telomeres in *stn1-13* $TLC1^+$ cells which during the same period of growth had become very long (lane 3). (B) Overexpression of *STN1* diminishes the *cdc13-276*-associated telomere elongation and aggravates the *cdc13-273*-associated telomere shortening. A *cdc13-1* YCp-*cdc13-273* mutant was transformed with YEp-*STN1*, a multicopy plasmid overexpressing *STN1* under the control of its natural promoter (lane 3), or with vector alone (lane 2). Both strains were grown at 32°C (to inactivate Cdc13-1), and the size of their telomeres were measured after approximately 100 generations. Short telomeres conferred by the Cdc13-273 protein (lane 2; compare to the telomere size in a *cdc13-1* strain grown at 25°C [lane 1]) were further shortened following *STN1* overexpression (lane 3). In addition, abnormal telomere lengthening conferred by the Cdc13-276 mutant protein (lane 4) was partially relieved when *STN1* was overexpressed (lane 5).

231 mutant proteins were only partially competent in binding telomeric DNA compared to wild-type Cdc13 (Fig. 2).

Telomere elongation in the telomere-elongating *cdc13* mutants requires both yKu70 and yKu80. We reasoned that the telomere-elongating Cdc13 mutant proteins might be deregulated in their interaction with another telomeric protein. To test this hypothesis, telomere-elongating alleles of *CDC13* were introduced into mutant strains known to exhibit abnormally short telomeres: *rad50*, *tel1*, *yku70*/*hdf1*, and *yku80*/*hdf2*. Rad50 is part of the Mre11-Rad50-Xrs2 telomeric complex that has been previously shown to function in so-called DNA nonhomologous end-joining (NHEJ) and in telomere maintenance (21, 28, 44). Tel1 is a telomeric protein that has been implicated in telomere length regulation and shares homology with the human ATM proteins (19, 35, 49). The yeast Ku proteins have been implicated in NHEJ, in heterochromatin organization, in telomere silencing, and in telomere maintenance (4, 7, 11–13, 18, 22, 37, 39).

Genetic disruption of either *RAD50*, *TEL1*, *YKU70*, or *YKU80* induced telomere shortening (Fig. 3), as previously demonstrated (3, 28, 35, 47). In the *cdc13-109i yku70*Δ and

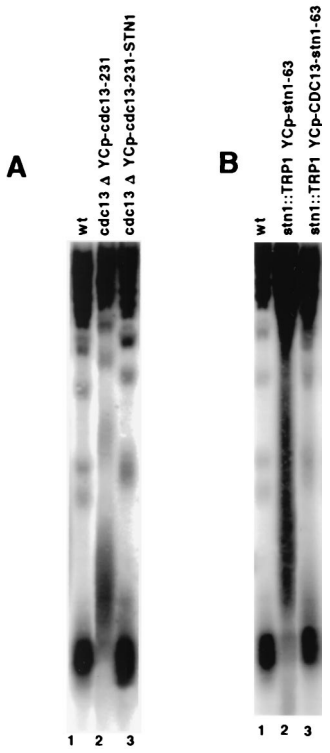

FIG. 7. Expression of Stn1-Cdc13 fusion proteins confirm that Stn1 negatively regulates Cdc13. (A) Expression of a *cdc13-231–STN1* hybrid gene from a single-copy centromeric plasmid under the control of the *CDC13* promoter in a *cdc13*Δ strain grown for approximately 100 generations totally suppressed the long telomere phenotype conferred by Cdc13-231 (compare lanes 2 and 3). (B) Expression of a *CDC13–stn1-63* hybrid gene in an *stn1*Δ strain under the same conditions as those described above totally suppressed the telomere lengthening phenotype conferred by Stn1-63 (compare lanes 2 and 3), which resulted in the acquisition of telomeres of wild-type size (lane 1).

*cdc13-109i yku80*Δ double mutants, telomeres were basically of the same length as those in the *yku70*Δ and *yku80*Δ single mutants (Fig. 3A, compare lane 7 to lane 8 and lane 14 to lane 15), whereas telomeres of the *cdc13-109i rad50*Δ double mutant were of an average length intermediate between those of each of the corresponding single mutants (Fig. 3A, compare lane 10 to lanes 11 and 12). The effect of *tel1*Δ in these experiments was between that of *yku70*Δ/*yku80*Δ and that of *rad50*Δ and was therefore more difficult to interpret (Fig. 3A, compare lane 1 to lanes 3 and 4). However, careful examination of the data revealed that the upper limit of the smear defining the average value of the bulk of telomere lengths was much higher in *cdc13-109i tel1* cells than in *cdc13-109i yku70* or *cdc13-109i yku80* cells (Fig. 3A). These observations were confirmed by experiments using the *cdc13-276* mutant (Fig. 3B).

A Cdc13-yKu70 fusion protein induces abnormal telomere lengthening. Because the genetic interactions described above were only indicative of a potential functional relationship between Cdc13 and either yKu70 or yKu80, we decided to adopt a complementary approach. One of our hypotheses to explain these interactions relied on the existence of a physical interaction between either yKu70 or yKu80 and Cdc13. Because attempts to detect physical association between Cdc13 and yKu70 in a two-hybrid system failed (unpublished results), we decided to use a method recently applied with success in the analysis of interactions between Cdc13 and Est1 (10), which consists in the expression of fusion (hybrid) proteins. We first expressed a Cdc13-Est1 fusion protein from a single-copy centromeric plasmid under the control of the *CDC13* promoter (see Materials and Methods) and observed that it produced telomere elongation in a *CDC13*$^+$ *EST1*$^+$ strain, an effect that was much more pronounced in a *cdc13-1* background at 34°C or in *cdc13*::*TRP1* than in a wild-type background (Fig. 4A, lanes 2, 8, and 11), as described recently (10).

We then analyzed the effects on telomere length of expressing a Cdc13-yKu70 fusion protein from a single-copy plasmid under the control of the *CDC13* promoter. The Cdc13-yKu70 fusion protein was fully functional because it rescued the nonviability of *cdc13*Δ cells as well as the growth defect of *yku70*Δ cells at 37°C (13) (data not shown). Expression of the Cdc13-yKu70 fusion resulted in telomere elongation in *cdc13*Δ and *CDC13*$^+$ cells, as well as in *cdc13-1* cells grown at 34°C (Fig. 4A, lanes 5, 9, and 12). In all backgrounds (*CDC13*$^+$, *cdc13-1*, and *cdc13*Δ), expression of the Cdc13-yKu70 fusion lengthened telomeres to a lesser extent than that of the Cdc13-Est1 fusion (Fig. 4A). Importantly, the Cdc13-yKu70 did not cause telomere elongation in a strain disrupted for *EST1* (Fig. 4B, lane 6). This suggested that the artificially introduced Cdc13-yKu70 hybrid protein affected telomerase recruitment via Est1. On the other hand, telomere elongation due to the Cdc13-Est1 fusion still took place in a strain disrupted for *YKU70* to the same extent as that in a *YKU70*$^+$ strain (Fig. 4A, compare lanes 3 and 4). In addition, expression of *CDC13* or *EST1* alone from a single-copy plasmid in a *cdc13-1 yku70*Δ strain (Fig. 4B, lanes 1 and 3) provided controls for the experiments shown above.

Because yKu70 and yKu80 are active in DNA repair only as a heterodimeric complex (12, 39), we measured the effect of expressing a Cdc13-yKu80 fusion protein on telomere length. Surprisingly, expression of a Cdc13-yKu80 fusion protein expressed from a single-copy centromeric plasmid under the control of the *CDC13* promoter, although it restored inviability of *cdc13-1* cells at 34°C, did not rescue the *yku80*Δ-induced telomere shortening (data not shown). We therefore constructed another hybrid protein which this time consisted of a yKu80-Cdc13 fusion protein expressed from a single-copy centromeric plasmid under the control of the *YKU80* promoter. The yKu80-Cdc13 fusion rescued the inviability of *cdc13*Δ cells but only partially the short telomere phenotype of *yku80*Δ (telomeres were of a heterogenous size, forming a smear whose upper limit reached the wild-type size and lower limit the *yku80*Δ size; data not shown). On the other hand, the yKu80-Cdc13 fusion produced only a moderate lengthening of telomeres in *cdc13*Δ cells (data not shown). Because of the lack of full functionality of the Cdc13-yKu80 and yKu80-Cdc13 fusions, one cannot conclude whether the absence of a drastic effect on telomere length of these fusions is real or not.

Finally, we asked whether the Cdc13-yKu70 fusion could produce telomere lengthening in the absence of yKu80. Interestingly, a *yku80*Δ *cdc13*Δ double mutant expressing the Cdc13-yKu70 under the conditions described above (see Fig. 4A) did not display any significant change in telomere length, unlike the *YKU80*$^+$ *cdc13*Δ YCp-Cdc13-yKu70 strain, which clearly exhibited telomere elongation (Fig. 4C, compare lanes 1 and 2). These experiments suggest that telomere elongation induced by the Cdc13-yKu70 fusion requires the presence of yKu80.

Novel nonsenescent telomere-shortening *cdc13* alleles are severely defective in DNA-binding activity. None of the telomere-shortening *cdc13* alleles described in this study (see Fig. 1A), namely, *cdc13-23*, *cdc13-30*, *cdc13-69*, *cdc13-243*, *cdc13-273*, and *cdc13-280*, provoked senescence (29, 33, 34) in contrast to *cdc13-2*/*est4-1* cells or *tlc1*Δ cells (data not shown). To further characterize these novel telomere-shortening Cdc13

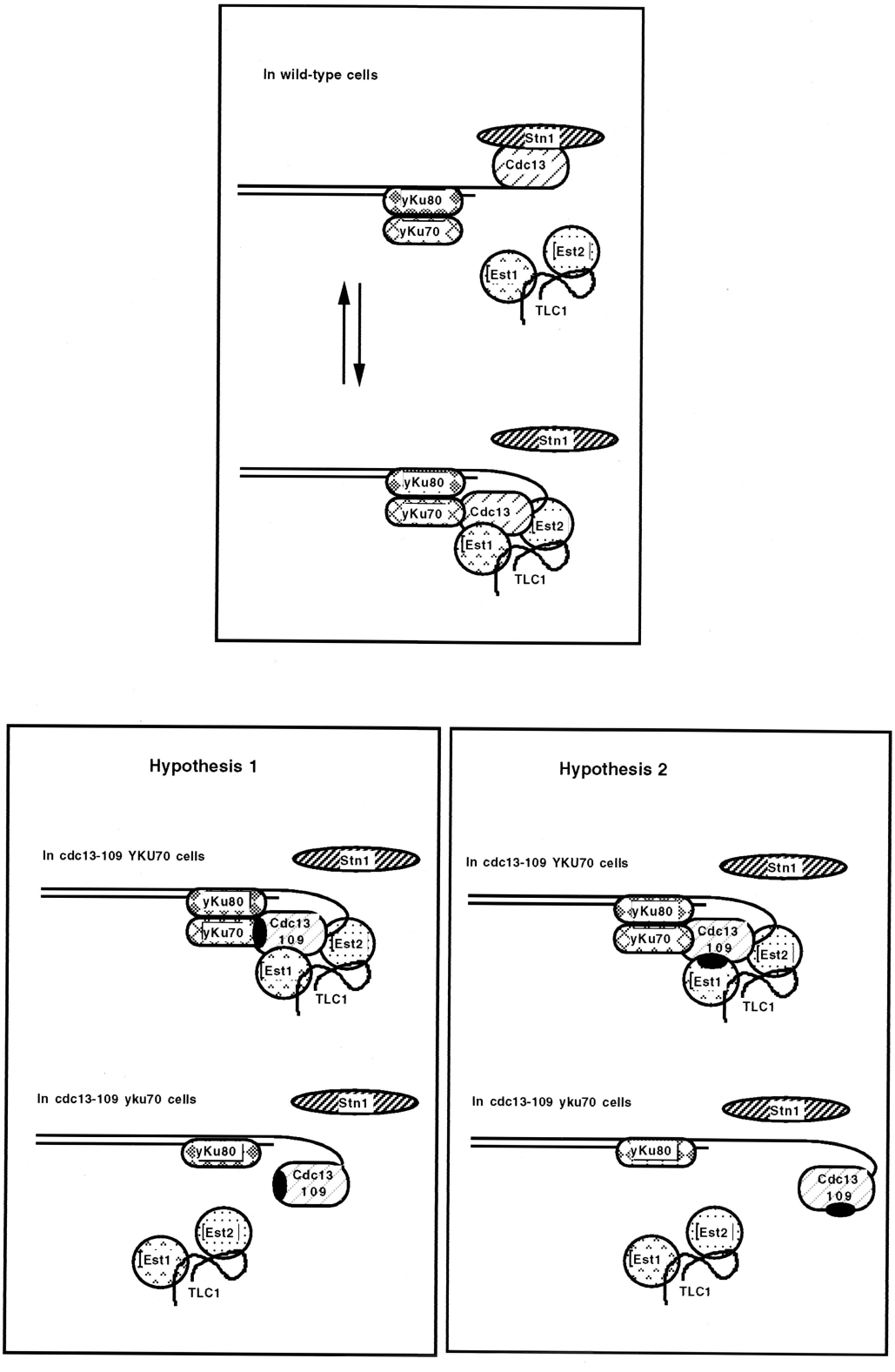

FIG. 8. (Top) A speculative model for yKu70 and Stn1 as positive and negative regulators, respectively, of Cdc13-Est1-mediated telomerase loading in wild-type cells. Stn1, which physically associates with Cdc13, a single-strand telomeric DNA-binding protein, acts as an inhibitor of Cdc13-mediated telomerase recruitment (telomerase contains the catalytic subunit, Est2, and *TLC1*, the RNA template; Est1, a regulator of telomerase which binds single-stranded telomeric DNA, also physically associates with *TLC1*). Stn1 release from the Cdc13-Stn1 complex might represent the signal allowing interactions between Cdc13 and Est1-telomerase. According to this model, interactions between Cdc13 and the DNA repair yKu proteins, yKu70 and yKu80 (yKu70 physically associates with yKu80, which itself binds double-strand telomeric DNA), might promote efficient association of Est1-telomerase with the telomere end, thus allowing telomeric DNA addition. Re-binding of Stn1 to Cdc13 might compete with Cdc13-yKu70 or -yKu80 and Cdc13-Est1 interactions, therefore breaking interactions between Est1-telomerase and telomeric DNA

mutant proteins, we performed band shift experiments to measure their ability to bind telomeric DNA (Fig. 5A). All six telomere-shortening Cdc13 mutant proteins were severely defective in binding telomeric DNA (Fig. 5A). Among these, Cdc13-69–2HA-6His Cdc13-243–2HA-6His, and Cdc13-273–2HA-6His retained some DNA binding activity, as confirmed by observing a supershifted band upon addition of anti-HA antibody during the reaction, while the Cdc13-23–2HA-6His, Cdc13-30–2HA-6His, and Cdc13-280–2HA-6His proteins were almost completely defective in binding telomeric DNA (Fig. 5A).

Among the six telomere-shortening *cdc13* alleles, *cdc13-69* conferred the largest telomere shortening effect (Fig. 1A). Integration of *cdc13-69* at the *CDC13* genomic locus (to create *cdc13-69i*) demonstrated that the Cdc13-69i protein could assume Cdc13's essential function (no apparent defect; data not shown) and conferred a short telomere phenotype similar in amplitude to that conferred by Cdc13-69 (compare lane 12 in Fig. 1A to lane 2 in Fig. 5C).

It has been shown that Cdc13-2, the only other Cdc13 mutant protein known to confer telomere shortening, was capable of binding telomeric DNA (45) and Est1 (48). It is important to note that a Cdc13-69–Est1 fusion protein conferred telomere lengthening, by a just slightly smaller degree than that produced by the Cdc13-Est1 fusion (Fig. 4A, lane 16), thus suggesting that increased association between Cdc13-69 and Est1 could cure the telomere size regulation defect of the Cdc13-69 mutant protein. However, because Est1 is also a single-strand telomeric DNA-binding protein (55), it is not possible yet to decide whether the defect of Cdc13-69 is in its ability to bind telomeric DNA, a defect rescued by Est1-mediated recruitment to DNA, or rather lies in a putative physical interaction with Est1. Expression of a Cdc13-69–yKu70 fusion also rescued the short telomere phenotype conferred by the *cdc13-69* allele (Fig. 4A, compare lanes 15 and 17), thus supporting the model that yKu70 promotes the Cdc13-mediated recruitment of telomerase.

Stn1 exerts a negative effect on Cdc13-mediated telomerase loading. We have previously proposed that Stn1 might negatively regulate Cdc13 activity (17). In view of the potential positive modulation of Cdc13 activity by yKu70, we speculated that Stn1 might feed negative signals into the Cdc13-regulating machinery. In our previous studies on Stn1 (17), it had not been demonstrated that a loss of Stn1 function led to deregulation of telomerase recruitment. We now demonstrate that the telomeric defect of *stn1-13*, a mutant which exhibits a severe growth defect at the restrictive temperature of 37°C and telomere elongation at any temperature between 25 and 37°C (17), results from a deregulation in telomerase recruitment and/or activity (Fig. 6A).

Because Stn1 loss of function leads to telomerase hyperactivation, Stn1, which physically associates with Cdc13 by two-hybrid interaction (17, 54), might be a negative regulator of Cdc13. If so, overexpression of *STN1* might be able to modify telomere length regulation. Overexpression of *STN1* from a multicopy (episomal, 2μ) plasmid under the control of its natural promoter produced no visible effect on telomere length in a wild-type strain (data not shown), as described previously (17). However, when *STN1* was overexpressed in a mutant strain expressing the telomere-shortening *cdc13-273* allele from a single-copy plasmid, a further increase in telomere shortening was observed (Fig. 6B, compare lanes 2 and 3). Likewise, overexpression of *STN1* in a mutant strain expressing the telomere-elongating *cdc13-276* allele from a single-copy plasmid resulted in a noticeable slowing down of telomere elongation (Fig. 6B, compare lanes 4 and 5). On the basis of these experiments, one can conclude that Stn1 behaves as an inhibitor of telomerase recruitment.

A possible mechanism accounting for the observations described above consists of direct titration of Cdc13 by Stn1. To test this prediction, we designed the following fusion protein experiments. Expressing a fusion protein made of Cdc13-231 and Stn1 in a *cdc13*Δ strain resulted in a dramatic suppression of telomere elongation conferred by the Cdc13 mutant protein (Fig. 7A, compare lanes 2 and 3). Thus, it appears that the defect of the Cdc13-231 protein in telomere length regulation can be totally corrected by providing a more permanent association between wild-type Stn1 and the Cdc13-231 mutant protein. As an important control, we verified that YCp111-*cdc13-231*-induced telomere elongation was not compromised by a fusion with Est1 (data not shown). Interestingly, a fusion between wild-type Cdc13 and wild-type Stn1 provoked a small shortening of telomeres (Fig. 4A, lane 13).

We next examined the consequences of fusing an Stn1 mutant protein conferring telomere elongation to wild-type Cdc13. To do this, we used *stn1-63*, a mutant that was generated using a PCR-based methodology (see Materials and Methods). The strain harboring *stn1-63* carried on a centromeric plasmid (YCp111-*LEU2*) in an *stn1* null background (*stn1*::*TRP1*) was selected on the basis of its deregulation in telomere length. The *stn1-63* mutant strain had no visible morphological or growth defect at temperatures between 25 and 37°C (data not shown). The constitutive defect in telomere size of *stn1-63* cells, namely, a very dramatic increase in telomere length (Fig. 7B, lane 2), is comparable to that in *stn1-13* cells growing at 34°C (17). As shown above for *stn1-13* (Fig. 6A), telomere lengthening conferred by *stn1-63* was found to depend entirely on telomerase (data not shown). Introduction of the gene encoding the Cdc13–Stn1-63 fusion carried by a single-copy plasmid under the control of the *CDC13* promoter into an *stn1*Δ strain almost totally suppressed telomere elongation conferred by Stn1-63 (Fig. 7B, compare lanes 2 and 3). This observation suggests that the defect of the Stn1-63 protein, which as far as we know about Stn1 function (17; present data) might be a failure to properly regulate Cdc13, can be almost totally corrected by artificially increasing its association with Cdc13 by means of a fusion protein.

and terminating the telomere replication process. (Bottom) Two hypotheses are proposed to explain the deregulation of telomere length conferred by the telomere-elongating *cdc13* alleles described in the present study (*cdc13-109*, which has been used in most of the experiments presented here, has been chosen for illustration). For both hypotheses, the situation has been envisioned either in the presence of the *cdc13-109i* mutation alone (cdc13-109 YKU70 cells) or in the simultaneous presence of the *cdc13-109i* and the *yku70*Δ mutations (cdc13-109 yku70 cells). Full ovals represent a higher than normal physical association between the deregulated Cdc13-109 mutant protein and yKu70 (hypothesis 1, left) or between Cdc13-109 and Est1 (hypothesis 2, right). In all of the configurations represented here, Stn1 is in the off position, physically apart from Cdc13, the position that presumably allows telomerase recruitment by Cdc13. In hypothesis 1 (left), constitutive interactions between Cdc13 and either yKu70 or yKu80 provokes recruitment of telomerase at higher than normal levels, thus leading to telomere lengthening (top), while the absence of yKu70 presumably results in inefficient recruitment of Est1-telomerase, thus suppressing *cdc13-109*-induced telomere lengthening (bottom). In hypothesis 2 (right), constitutive interactions between Cdc13 and Est1 also provoke recruitment of telomerase at higher-than-normal levels and leads to telomere lengthening (top), but this time the absence of yKu70 presumably generates an abnormal single-stranded telomeric DNA extension which competes for Cdc13-109–Est1 interactions and results in inefficient recruitment of Est1-telomerase and suppression of the *cdc13-109*-induced telomere lengthening (bottom). See the text for further explanations.

Sequence analysis of the *cdc13* alleles. DNA sequencing revealed that all six sequenced *cdc13* alleles (*cdc13-7*, *cdc13-69*, *cdc13-109*, *cdc13-243*, *cdc13-273*, and *cdc13-276*) contained multiple point mutations. In addition, a mutation in lysine 706 of Cdc13-276 introduced a termination codon that resulted in the truncation of the last 218 amino acids, while a frameshift in *cdc13-7* sequence at lysine 702 led to the introduction of a premature stop codon at amino acid 721. We have not been able so far to identify the mutations responsible for the phenotypes of the corresponding mutants.

DISCUSSION

The survival of an organism relies on the proper duplication, segregation, and stability of its genome. Telomeres play an important role in maintaining chromosome structure because, for instance, chromosomes that lose a telomere are themselves eliminated from the cell (50). In yeast, mutations in telomerase components or regulators produce a gradual erosion of chromosomes that eventually leads to death by senescence due to chromosome instability (29, 34). Cdc13 has been previously implicated, together with Est1, as the main regulator of telomerase access to telomeric ends (10, 30, 45, 48, 61).

The present study extends our knowledge on the role of Cdc13 in telomerase control through the analysis of novel *cdc13* alleles and their functional relationships with mutations in other telomeric proteins. Our data suggest that the yeast Ku proteins, previously implicated in DNA repair and telomere maintenance in yeast and humans (4, 7, 16, 24, 27, 37, 39), promote telomerase recruitment by Cdc13. Our results also demonstrate that regulation by yKu70 or yKu80 is opposite of that by Stn1, since we found that Stn1 negatively regulates Cdc13-mediated telomerase recruitment. The present study also provides telomere-shortening *cdc13* alleles that do not confer senescence and may be useful in some biochemical or genetic assays.

Stn1 inhibits telomerase recruitment via Cdc13. Our observation that *STN1* overexpression produces telomere shortening (Fig. 6B) is reminiscent of the effects of overexpressing *RIF1* or *RIF2* (59). A striking parallel between these two situations is that Rif1 and Rif2 physically associate with Rap1, a master regulator of telomere length which binds double-stranded telomeric DNA (23, 36, 59), while Stn1 physically associates with Cdc13, a master regulator of telomere length which binds single-stranded telomeric DNA (5, 17, 26, 30, 45). However, a noticeable difference between the two situations was that *STN1* overexpression did not affect telomere length in wild-type cells (17; the present data), whereas overexpression of *RIF1* and *RIF2* did (59). We propose that overproduction of Stn1 can affect telomere length only when Cdc13 function is already compromised, as explained below.

Experiments using fusion proteins further established the role of Stn1 as an inhibitor of Cdc13's telomerase loading function (Fig. 7). As argued from experiments using Cdc13-Est1 fusion proteins (10), the present data suggest the existence of physical interactions between Cdc13 and Stn1. Indeed, suppression of the *cdc13-231*-induced telomere elongation following consolidation of its natural interaction with Stn1, as well as suppression of the *stn1-63*-induced telomere elongation following consolidation of its natural interaction with Cdc13, strongly argue that a major control over Cdc13 activity operates through physical association with Stn1. In fact, because it is already known that Stn1 and Cdc13 associate in a two-hybrid system (17, 54), the experiments on Cdc13-Stn1 fusions presented here enhance interpretations made concerning experiments done with the Cdc13-yKu70 fusion (Fig. 4). It is noticeable that a fusion made of wild-type Cdc13 and wild-type Stn1 provoked a small shortening of telomeres (Fig. 4A, lane 13), the interpretation of which is discussed below.

yKu70 promotes the recruitment of telomerase by Cdc13. Disrupting either *YKU70* or *YKU80* had a larger suppressing effect on telomere elongation conferred by the Cdc13-276 or Cdc13-109 mutant proteins than disrupting *RAD50* or *TEL1* (Fig. 3). Although at first glance *tel1*Δ may appear to have an effect similar to that of *yku70*Δ or *yku80*Δ in diminishing the *cdc13-109*-associated telomere elongation, careful examination of the Southern blot revealed a difference between the two, which was confirmed in the *cdc13-1* YCp-*cdc13-276* strain. It could be argued that such experiments are difficult to interpret because, for instance, all of the telomere-shortening mutations considered here are in proteins known to be involved each in several pathways, including telomere maintenance. Moreover, since the two mutations present in a given strain affect telomere length in opposite directions, it is difficult to determine whether the resulting average telomere length corresponds to equilibrium between the two opposing effects or rather reflects actual genetic interaction between the two mutations. For these reasons, we were very cautious in interpreting these epistasis experiments. In the end, a noticeable result is that the *yku70*Δ and *yku80*Δ mutations totally suppressed the *cdc13-109*-induced telomere elongation (Fig. 3A), while conferring wild-type length telomeres to strains bearing the *cdc13-276* mutation (Fig. 3B). The effects of *rad50*Δ and *tel1*Δ were smaller than those of *yku70*Δ and *yku80*Δ in either *cdc13* strain.

Because we did not want to overinterpret the epistasis experiments discussed above, we used these results only as an indication and not as a conclusive argument. In fact, the indications provided by these epistasis experiments were further confirmed by the finding that expression of a Cdc13-yKu70 fusion protein clearly resulted in telomere lengthening (Fig. 4). Given that telomere elongation in the *cdc13* alleles described here depends on Est1 (Fig. 1C), altogether these findings suggest that yKu70 and yKu80 may regulate interactions between Cdc13 and telomerase. Importantly, the presence of yKu80 was necessary to mediate the effect of the Cdc13-yKu80 on telomere length (Fig. 4C). A possible model is that yKu70 might help recruit telomerase through an interaction with Cdc13, as explained below, with yKu80 playing a crucial role in this mechanism as representing the Ku component attached to telomeric DNA.

A model for the control of Cdc13-mediated telomerase recruitment by Stn1 and by the yKu70 and yKu80. Based on the experiments presented here and the data available in the literature (principally, references 4, 10, 14, 17, 18, 25, 26, 29, 30, 40, 44–48, 55, 57, and 61), we propose an improved model for the control of telomerase recruitment by Cdc13 that involves the existence of a balance between the effects of yKu70 and Stn1 on Cdc13 (Fig. 8). The top panel of Fig. 8 depicts the situation in wild-type cells, while the bottom panels propose two hypotheses to account for the situation encountered in the *cdc13-109i* mutant strain.

According to our model (Fig. 8, top panel), one might expect Stn1 overproduction to shorten telomeres in wild-type cells, which was not observed experimentally. In fact, this result can be explained by assuming that Cdc13 is solidly anchored at telomere ends and that overproduced Stn1 cannot titrate it out or pull it away from the telomeres. Under such conditions, only the presence of Stn1, but not its amount, in close proximity to Cdc13 would be required for the precise tuning of telomere length control, according to our working model (Fig. 8, top panel). On the other hand, a Cdc13 mutant protein exhibiting an altered interaction with another telomeric protein (as de-

picted, for instance, for Cdc13-109 in Fig. 8, bottom panels) or telomeric DNA (as is the case for Cdc13-69; see Fig. 5A), might see its overall stability affected, thus resulting in competitive inhibition by Stn1 for the affected binding sites and, hence, in telomere shortening (Fig. 6B). Confirmation of this view must await experimental support.

We observed that expression of a fusion between wild-type Cdc13 and wild-type Stn1 resulted in a slight shortening of telomeres (Fig. 4A, lane 13). It is difficult to know whether such a decrease is significant or not. If it is significant, then expressing a single copy of the Cdc13-Stn1 fusion gene would be more efficient on Cdc13 regulation than overexpressing *STN1*. This point of view is supported by the fact that overexpression of *STN1* only slightly affected telomere length in *cdc13* mutant strains (Fig. 6B), whereas expressing a fusion between a Cdc13 mutant protein and wild-type Stn1 had a very dramatic effect on telomere length (Fig. 7A). Although these effects are compatible with the model proposed here, it is too early to provide an accurate explanation of the molecular mechanisms involved, particularly since we know that the defects of the Cdc13 mutant proteins described here are still at the hypothetical stage (Fig. 8, bottom panels). In addition, full comprehension of these mechanisms may be complicated by the possible existence of still-unknown partners of Cdc13 and Stn1.

Because both the Cdc13-yKu70 and the Cdc13-Est1 fusions produced telomere elongation that mimicked telomere deregulation in the *cdc13* mutants, the corresponding Cdc13 mutant proteins may be deregulated in their interaction with either yKu70 or Est1 (Fig. 8, bottom panels). On first analysis, a potential defect in the association of Cdc13 with yKu70 in these *cdc13* mutants is the more plausible explanation (hypothesis 1, Fig. 8, left bottom panel). Indeed, in the alternative hypothesis—a defect in the association of Cdc13 with Est1 in these *cdc13* mutants—one should not observe a suppressing effect of the *yku70*Δ and *yku80*Δ mutations (Fig. 3) unless the yKu proteins directly interact with telomerase. However, a defect in Cdc13-Est1 interactions could fit with some other aspects of Ku functions (hypothesis 2, Fig. 8, right bottom panel). Indeed, yeast Ku mutants have been shown to retain extended TG_{1-3} tails throughout the cell cycle (46), unlike wild-type cells which retained them only during late S phase (57). It has been proposed that the yeast Ku proteins are responsible for controlling the 5′-3′ processing of telomere ends (22, 46, 56). Therefore, the absence of the Ku proteins might structurally modify the telomeric ends so as to produce an alteration of Cdc13 positioning on the 3′ free end. Under such conditions, disruption of *YKU70* or *YKU80* in the *cdc13-109i* mutant might result in Cdc13-109 being physically displaced along the telomere end due to the presence of longer single-stranded telomeric sequences, which might then, by competitive interactions, abolish the higher than normal association between Cdc13-109 and Est1. These hypotheses should now be challenged by biochemical experiments.

Even though the details of the interactions described here have to be elucidated in future experiments, our data establish two major conclusions. First, expression of a Cdc13-yKu70 fusion promotes telomerase recruitment, which is supported by the finding that, in the absence of either Ku protein, the Est1-dependent recruitment of telomerase by Cdc13 is compromised. Second, Stn1 exerts a negative control over Cdc13-mediated telomerase recruitment.

Stn1 and Rif1 or Rif2 might exert two parallel and complementary levels of negative control of telomere length, taking place at two spatially different locations on the telomere. It has been suggested that the Rap1-dependent telomere length-sensing mechanism (36) might be mediated by a balance between opposing effects of Rif1 or Rif2 and those of Sir3 or Sir4 (59). In light of the present data, as well as of the recent demonstration of a competition between Rif1 or Rif2 and yKu70 in the recruitment of Sir proteins by Rap1 at the telomere (40) and of the localization of the Ku proteins potentially at the junction between double-stranded and single-stranded telomeric DNA (18, 37), the Ku proteins represent likely candidates for constituting a functional link between Rap1, Rif1, and Rif2 and Cdc13 that is capable of modulating telomerase recruitment. If our hypothesis is correct, the Ku proteins might contribute to control telomere length by their ability to detect changes in telomere structure, or even by an ability to modify telomere structure (46), parameters that would then be fed into the Cdc13 machinery according to mechanisms proposed above. On the other hand, Stn1 might perceive and convey other types of signals, possibly emanating from extratelomeric locations. The genetic system described here, based on the analysis of yeast Cdc13 mutant proteins and their interaction with other wild-type or mutant telomeric proteins, provides an excellent frame with which to study the mechanism of telomerase recruitment at the telomeres.

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